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## Pharmacokinetics and Drug Metabolism

## ABCC4 is regulated by microRNA-124a and microRNA-506



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## ABSTRACT

Multidrug resistance protein 4 (MRP4, ABCC4) is an efflux membrane transporter expressed in renal tubules, hepatocytes, brain capillaries, prostate and blood cells. MRP4 drives energy dependent efflux of important physiological and pharmacological compounds. MRP4 expression and function is highly variable but cannot be fully attributed to known mechanisms. The goal of this study was to characterize ABCC4 regulation by miRNAs and to assess the influence of ABCC4 3'-UTR polymorphisms on ABCC4 regulation by miRNAs. miR-124a and miR-506 decreased MRP4 protein levels in HEK293T/17 cells 20–30% and MRP4 function by 50%. These miRNAs did not affect ABCC4 mRNA expression. Moreover, miR-124a and miR-506 expression was negatively correlated with MRP4 protein expression in 26 human kidney samples (Spearman  $r = -0.62$ ,  $P = 0.007$  and  $r = -0.41$ ,  $P = 0.03$  for miR-124a and miR-506, respectively). To assess the effect of ABCC4 3'-UTR polymorphisms, six common 3'-UTR haplotypes were inferred in Caucasians, African Americans and Asians and tested in luciferase reporter assays. Multiple ABCC4 3'-UTR haplotypes caused significant reductions in luciferase activity; in the presence of miR-124a or miR-506 mimics the luciferase activity of all six ABCC4 3'-UTR haplotypes was further reduced. Mutation of the putative binding site for miR-124a and miR-506 in the ABCC4 3'-UTR eliminated the effect of these miRNAs. In conclusion, ABCC4 is directly regulated by miR-124a and miR-506 but polymorphisms in the ABCC4 3'-UTR have no significant effect on this miRNA regulation. Regulation of ABCC4 by miRNAs represents a novel mechanism for regulation of MRP4 function.

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## 1. Introduction

The multidrug resistance protein 4 (MRP4), encoded by *ABCC4*, is a member of the ATP-binding cassette (ABC) family of membrane transporters. MRP4 is expressed in a variety of tissues and can be localized both apically (renal proximal tubule cells, luminal side of brain capillary endothelium) and basolaterally (prostate tubuloincinar cells and hepatocytes). It is also detected in epithelial cells of seminal vesicles, ovary, adrenal gland, dendritic cells, erythrocytes, monocytes and delta granules of platelets [1,2]. MRP4 plays an important role in the pharmacokinetics of a wide variety of endogenous and exogenous compounds. This transporter drives energy dependent efflux of such physiologically relevant compounds as cyclic nucleotides, ADP, prostaglandins, leukotrienes, taurine and glycine conjugates of bile acids, and glucuronide conjugates of steroid hormones. It also transports a broad spectrum of xenobiotics including antiviral drugs (adefovir,

tenofovir), antibiotics (ceftizoxime, cefmetazole), antihypertensive drugs (furosemide, hydrochlorothiazide, olmesartan) and anticancer agents (methotrexate, 6-thioguanine, 6-mercaptopurine, topotecan) [2,3]. The importance of MRP4-mediated transport of endogenous and therapeutic compounds has been clearly demonstrated in mouse models lacking *Mrp4* expression [4–6].

MRP4 expression and function is highly variable. It has been associated with the onset and prognosis of several diseases [7–9] as well as drug efficacy and toxicities [10,11]. A number of common *ABCC4* promoter and non-synonymous coding region polymorphisms have been associated with MRP4 expression and/or function [12–14] and with drug disposition, response and adverse events [15–17]. Currently, ABCC4 regulation cannot be fully explained or attributed to known *ABCC4* polymorphisms [18].

In the present study we focused on ABCC4 regulation by miRNAs. miRNAs are 18–25 nucleotide long non-coding RNAs regulating gene expression. They are generated from endogenously transcribed primary miRNA (pri-miRNA) hairpin structures, which are further cleaved in the cell nucleus by Drosha (RNase III) [19], yielding a 70–100 nucleotide long stem loop precursor miRNA (pre-miRNA). Pre-miRNAs are trafficked from the nucleus to the cytoplasm by Exportin 5 [20] and are further processed by Dicer (RNase III) [21] to produce mature miRNA duplexes that are 18–25

Abbreviations: MCB, (monochlorobimane); ABCC4, (ATP-binding cassette transporter member 4); MRP4, (multidrug resistance protein member 4).

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nucleotides in length. The antisense miRNA strand is then incorporated into a RNA-induced silencing complex (RISC) where it binds to a complementary sequence of 3'-UTR. The "seed", 2–9 nucleotides at the 5' end of miRNA, is crucial for binding to target mRNA. Upon binding, the miRNA initiates translational repression or cleavage of targeted mRNA [22,23].

A significant number of miRNAs have been discovered and annotated, although only a small portion have been functionally validated. miRNAs are currently implicated in the regulation of metabolic enzymes (CYP3A4 and CYP1B1) [24,25] as well as drug transporters (ABCB1, ABCB6, ABCC1, ABCC2, ABCC4, ABCC5, ABCC10, ABCC12 and ABCG2) [26–34] and it is likely that they are involved in the regulation of drug response. The present study was designed to characterize ABCC4 regulation by predicted miRNAs and to assess the influence of ABCC4 3'-UTR genetic polymorphisms on ABCC4 regulation by miRNAs.

## 2. Materials and methods

### 2.1. *In silico* predictions of miRNAs targeting ABCC4

We used multiple web-based tools designed to predict miRNA targets, namely miRBase (<http://www.mirbase.org/>), PicTar (<http://pictar.mdc-berlin.de/>), Segal Lab tool (<http://genie.weizmann.ac.il/pubs/mir07/index.html>), miRDB (<http://mirdb.org/miRDB/>), miRNAmap (<http://mirnamap.mbc.nctu.edu.tw/>), miRacle (<http://miracle.igib.res.in/miracle/>), miRTar (<http://mirtar.mbc.nctu.edu.tw/human/>), and DIANA Lab (<http://diana.cslab.ece.ntua.gr/>). All of these tools take into account complementarity of the miRNA "seed", miRNA binding site conservation, and energy of binding to miRNA. The Segal Lab tool miRNA prediction algorithm also considers the energy of mRNA unwinding, thus accounting for mRNA secondary structure. miRNAs predicted by at least two independent tools were selected for further testing.

### 2.2. Reagents and miRNA mimics

All miRNA mimics and ABCC4 siRNA were purchased from Applied Biosystems (Foster City, CA, USA). Monochlorobimane (MCB) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.3. Human kidney samples

Twenty six healthy human kidney samples were purchased from Capital Biosciences, Gaithersburg, MD, USA.

### 2.4. Cell culture

Human embryonic kidney (HEK293T/17) cells (from American Tissue Culture Collection, ATCC, Manassas, VA, USA) were cultured in complete DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### 2.5. Transfection of mimic miRNAs into HEK293T/17 cells

HEK293T/17 cells were seeded in 24 well plates at a density of  $1 \times 10^5$  cells/well, allowed to attach and then transfected with 5, 20, 50 or 100 nM of miRNA mimics. All transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Seventy two hours post transfection cells were washed with PBS and lysed on ice for 10 min with CellLytic reagent (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were then centrifuged at 4 °C at 10,000g for 10 min and the supernatant was collected. Protein concentrations were measured using a BCA assay (Thermo Scientific, Rockford, IL, USA) and samples were frozen at –80 °C until MRP4 protein detection.

### 2.6. Protein isolation and Western blot analysis

Proteins were isolated using mirVANA™PARIS™ kit (Ambion by Life Technologies, Foster City, CA, USA) according to the manufacturer's protocol. Proteins (30 µg/lane) were separated on 4–15% Tris–HCl Criterion gels (Bio-Rad, Hercules, CA, USA) by SDS-PAGE at 80 V and then transferred onto nitrocellulose membranes at 250 mA for 2 h. Membranes were blocked in 2% skim milk for 1 h at room temperature and incubated overnight at 4 °C with anti-MRP4 antibody (M4I-10) at 1:250 dilution (Alexis Biochemicals, San Diego, CA, USA) or anti-GAPDH antibody (ab9483) at 1:100,000 dilution (Abcam, Cambridge, MA, USA). The following day, membranes were washed three times with PBS and then incubated with IRDye 800CW goat anti-rat (anti-MRP4) or anti-mouse (anti-GAPDH) fluorescent secondary antibody (LI-COR, Lincoln, NE, USA) at 1:10,000 dilution. Membranes were washed with PBS and visualized on an Odyssey® Sa Infrared Imaging System (LI-COR, Lincoln, NE, USA).

### 2.7. RNA isolation and real time quantitative RT-PCR (qRT-PCR) analysis

Total RNA enriched in small RNAs was isolated using mirVANA™PARIS™ kit (Ambion by Life Technologies, Foster City, CA, USA) according to the manufacturer's protocol. To obtain cDNA for ABCC4 and GUSB measurements, 500 ng of total RNA were reverse transcribed in a 10 µL reaction using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To obtain cDNA for microRNA measurements, 20 ng of total RNA were reverse transcribed in a 20 µL reaction using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's multiplex protocol with slight modifications. Specifically, each reaction contained 2 µL of each original 5X RT primer set (Applied Biosystems, Foster City, CA, USA; assay ID numbers: 001182, 001050, 001093, 001006 for miR-124a, miR-506, RNU6B and RNU48, respectively), 0.4 µL of 100 mM dNTPs, 2 µL of reverse transcriptase (50 U/µL), 2 µL of 10X RT buffer, 0.25 µL of RNase inhibitor (20 U/µL) and water up to 20 µL. ABCC4, GUSB and microRNA PCR reactions contained 1 µL of cDNA, 5 µL of 2X SensiFAST™ HiROX mix (Bioline Reagents Ltd., Boston, MA, USA) and 0.5 µL of one of the primer/probe mixes (Applied Biosystems, Foster City, CA, USA; assay ID numbers Hs00195260\_m1, Hs99999908\_m1, 001182, 001050, 001093, 001006 for ABCC4, GUSB, miR-124a, miR-506, RNU6B and RNU48, respectively). Real time quantitative PCR measurements were performed on a HT7900 Real Time Fast PCR System (Applied Biosystems, Foster City, CA, USA). ABCC4 mRNA expression was expressed relative to GAPDH mRNA expression, miR-124a and miR-506 levels were expressed relative to geometric mean of RNU6B and RNU48 RNA expression using the  $2^{-\Delta\Delta Ct}$  method [35].

### 2.8. MRP4 transport assay

Seventy two hours after transfection with negative miRNA, siRNA, miR-124a and miR-506 as described above the efflux of bimane-glutathione, a metabolite of monochlorobimane (MCB) and a MRP4 substrate, was measured with slight modifications as previously described [36]. Briefly, the cells were washed with warm PBS and then incubated with 100 µM MCB in DMEM at 10 °C for 60 min. The plate was placed on ice and after washing with cold Hank's balanced salt solution (HBSS) the cells were incubated at 37 °C in HBSS containing 5.6 mM glucose for 15 min. Incubation buffer was collected for bimane-glutathione measurements. The fluorescence of collected samples was read using an excitation wavelength of 385 nm and an emission

wavelength of 478 nm using Magellan software (Tecan, Männedorf, Switzerland). The cells were washed with PBS and lysed on ice for 10 min with CellLytic reagent (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were then centrifuged at 4 °C at 10,000g for 10 min and supernatant was collected. Protein concentrations were measured using a BCA assay (Thermo Scientific, Rockford, IL, USA). Data was processed as relative fluorescence units corrected for total protein and presented as percent of microRNA negative control.

## 2.9. Construction and mutagenesis of ABCC4 3'-UTR haplotypes in luciferase reporter plasmids

The 3'-UTR of ABCC4 was amplified from human liver cDNA and was inserted downstream of the luciferase gene in the pGL3 promoter plasmid (Promega Corporation, Madison, WI, USA). Site-directed mutagenesis to obtain ABCC4 3'-UTR haplotypes was performed using a Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol.

## 2.10. Luciferase assays

HEK293T/17 cells were seeded on 96 well plates at a density of  $5 \times 10^4$  cells/well. The pGL3 promoter empty vector or plasmids containing ABCC4 3'-UTR reference and variant haplotypes were co-transfected with negative miRNA, siRNA, miR-124a or miR-506 together with *Renilla* luciferase vector into HEK293T/17 cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). *Renilla* luciferase vector served as a transfection efficiency control. Cells were incubated for 24 h at 37 °C in humidified atmosphere with 5% CO<sub>2</sub>, after which they were washed with phosphate buffered saline and incubated with 80 µL of passive lysis buffer (Promega, Madison, WI) at room temperature on a shaker for 30 min. Aliquots were analyzed for luciferase activity in a dual luciferase reporter assay on a GloMax™ 96 luminometer (Promega, Madison, WI, USA) according to the manufacturer's instructions.

## 2.11. Statistical analysis

All miRNA transfection results were normalized to the values obtained from samples transfected with negative miRNA. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni correction.  $P < 0.05$  was considered statistically significant. For microRNA and MRP4 correlations in human kidney samples, MRP4 protein densitometric measurements were expressed relative to the mean of all samples. microRNA expression data were natural logarithm transformed to obtain a normal distribution. Pearson correlation coefficients were calculated and corresponding  $P$  values  $< 0.05$  were considered statistically significant.

## 3. Results

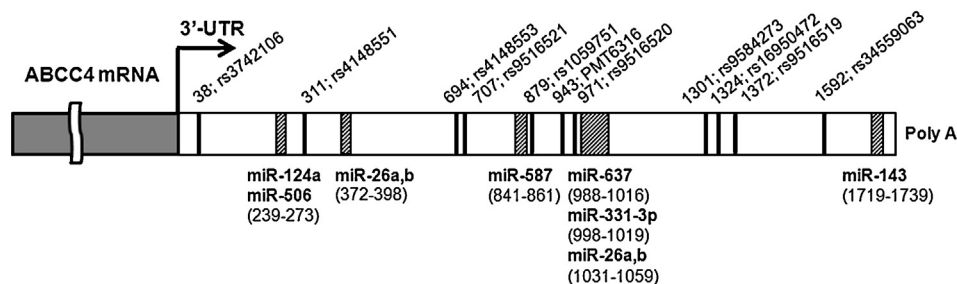
### 3.1. miRNA predictions

Using web-based miRNA prediction tools eight miRNAs (hsa-miR-26a, hsa-miR-26b, hsa-miR-124a, hsa-miR-143, hsa-miR-331-3p, hsa-miR-506, hsa-miR-587 and hsa-miR-637) were predicted to target the 3'-UTR of ABCC4 mRNA. The location of these predicted binding sites in the ABCC4 3'-UTR is illustrated in Fig. 1.

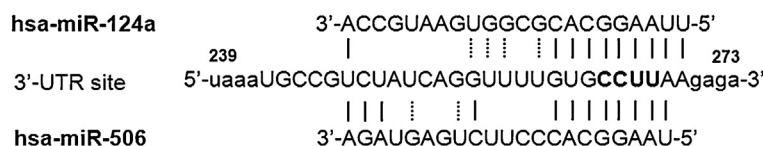
### 3.2. Effect of predicted miRNAs on MRP4 expression

To investigate if predicted miRNAs had an effect on MRP4 expression, HEK293T/17 cells were transfected with 50 nM of either negative control miRNA, siRNA against ABCC4 or predicted miRNAs. siRNA against ABCC4 significantly downregulated MRP4 protein in HEK293T/17 cells. Moreover, two out of eight tested miRNAs, hsa-miR-124a and hsa-miR-506, significantly downregulated MRP4 protein ~20–30% (Fig. 2). miR-124a and miR-506 were further tested for concentration dependent effects. HEK293T/17 cells were transfected with 5, 20, 50 or 100 nM of negative control, miR-124a or miR-506. Both miRNAs tended to downregulate MRP4 protein in HEK293T/17 cells in a concentration dependent manner (Fig. 3), although the trend did not reach statistical significance.

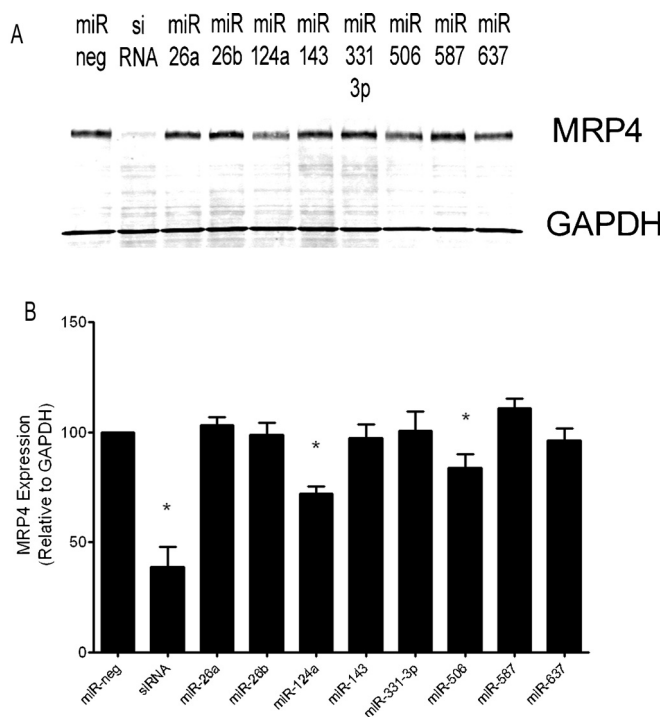
### A) Polymorphisms and microRNA response elements (MRE) in ABCC4 3'-UTR



### B) Putative binding site for miR-124a and miR-506 in ABCC4 3'-UTR



**Fig. 1.** Schematic representation of human ABCC4 mRNA with locations of 3'-UTR polymorphisms and putative microRNA response elements (MRE). (A) The numbering begins at the start of the 3'-UTR. Polymorphisms are shown as single lines throughout the ABCC4 3'-UTR, with position and rs number indicated. The location of putative response elements are indicated with hatched areas and the specific miRNAs are noted. (B) Sequences of miR-124a and miR-506 and their binding to a putative MRE in ABCC4 3'-UTR (239–273) are shown. Four nucleotides (CCUU) in ABCC4 3'-UTR, which were mutated in functional experiments, are shown in bold.



**Fig. 2.** Effect of miRNA mimics on endogenous MRP4 expression in HEK293T/17 cells. Cells were transfected with 50 nM of negative miRNA, siRNA against ABCC4 or predicted miRNA mimics. MRP4 protein expression was measured 72 h post transfection. (A) A representative Western blot is shown. (B) Densitometry results are from at least three independent experiments and are presented as mean ( $\pm$  S.D.). MRP4 levels are expressed relative to GAPDH; negative miRNA is set at 100%. MRP4 expression in the presence of miRNA mimics was compared to negative miRNA by one-way ANOVA, followed by Bonferroni correction; \*  $P < 0.05$ .

### 3.3. Effect of miR-124a and miR-506 on ABCC4 mRNA expression

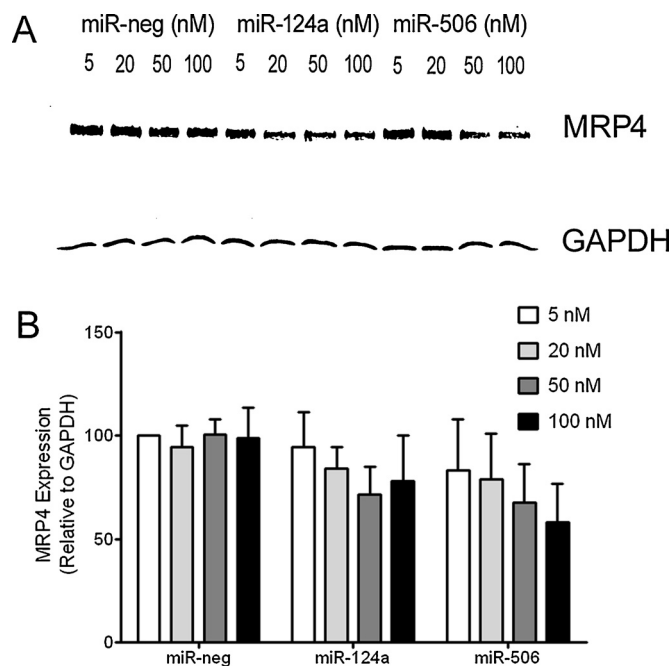
To investigate whether miRNA regulated MRP4 at the mRNA level, ABCC4 mRNA levels were measured in HEK293T/17 cells 24, 48 and 72 h after transfection with 50 nM of either negative control miRNA, siRNA against ABCC4, miR-124 or miR-506. siRNA against ABCC4 significantly reduced ABCC4 mRNA expression at all of the tested time points while miRNAs only modestly reduced ABCC4 mRNA expression (Fig. 4).

### 3.4. Effect of miR-124a and miR-506 on MRP4 function

To investigate whether the reduction in MRP4 protein expression had corresponding effects on MRP4 function, HEK293T/17 cells were transfected with 50 nM negative control miRNA, siRNA against ABCC4, miR-124a or miR-506 and a transport assay with monochlorobimane (MCB) was performed 72 h post transfection. MCB diffuses into cells and is metabolized into the fluorescent conjugate, bimane-glutathione, which is a MRP4 substrate [36]. Efflux of bimane-glutathione was measured to assess MRP4 function. Both miR-124a and miR-506 significantly reduced MRP4 transport activity almost 50% (Fig. 5). Interestingly, the effect of siRNA on MRP4 function was very comparable to the effects of miRNAs (Fig. 5), despite significant differences in their effects on MRP4 protein expression (Fig. 1).

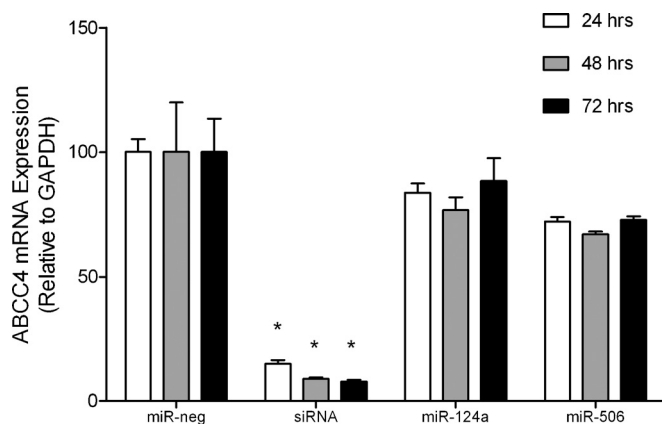
### 3.5. Characterization of ABCC4 3'-UTR haplotypes

The ABCC4 3'-UTR region is highly polymorphic with a number of SNPs in close linkage disequilibrium. The location of the 3'-UTR SNPs that were investigated is shown in Fig. 1. ABCC4 3'-UTR



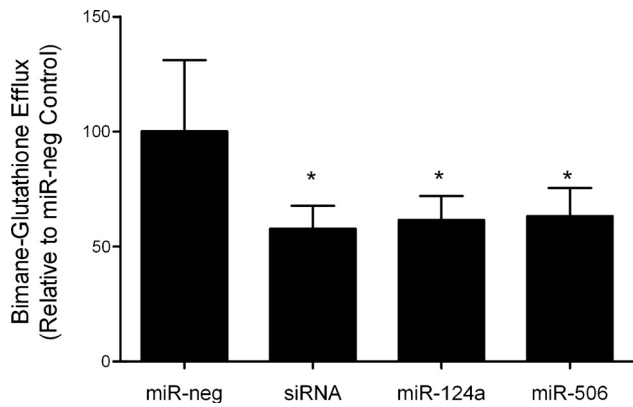
**Fig. 3.** Dose dependent downregulation of endogenous MRP4 in HEK293T/17 by miR-124a and miR-506 mimics. Cells were transfected with 5, 20, 50 or 100 nM of negative miRNA, miR-124a or miR-506. MRP4 protein expression was measured 72 h post transfection. (A) A representative Western blot is shown. (B) Densitometry results are from at least three independent experiments and are presented as mean ( $\pm$  S.D.). MRP4 levels are expressed relative to GAPDH; negative miRNA is set at 100%. MRP4 expression at each concentration of miRNA mimic was compared to the corresponding negative miRNA control by one-way ANOVA, followed by Bonferroni correction but did not reach statistical significance.

haplotypes were predicted in three ethnic groups (Caucasians, African Americans and Asians) using HapMap and Pharmacogenomics of Membrane Transporters (PMT) genotyping data. Six haplotypes (H1-H6) were inferred with a frequency of more than 5% (Table 1); each haplotype contained at least two common polymorphisms. H1 and H2 were present at a high frequency in all three ethnic groups and can both be considered the reference



**Fig. 4.** Effect of siRNA, miR-124a and miR-506 on ABCC4 mRNA expression in HEK293T/17 cells. Cells were transfected with 50 nM of negative miRNA, siRNA against ABCC4, miR-124a and miR-506. ABCC4 mRNA expression was measured 24, 48 and 72 h post transfection by qRT-PCR. mRNA levels are expressed relative to GAPDH levels. Results from one representative experiment ( $n = 3$  independent experiments) are presented as mean  $\pm$  S.D. (3 replicates/condition) with negative miRNA set at 100%. Differences relative to negative miRNA were analyzed by one-way ANOVA, followed by Bonferroni correction; \*  $P < 0.05$ .





**Fig. 5.** Effect of siRNA, miR-124a and miR-506 on transport of bimeane-glutathione by MRP4. HEK293T/17 cells were transfected with 50 nM negative miRNA, siRNA, miR-124a and miR-506. After 72 h, cells were incubated with monochlorobimane (MCB) and bimeane-glutathione efflux was measured. Bimeane-glutathione fluorescence was normalized to total protein and results from one representative experiment ( $n = 3$  independent experiments) are presented as mean  $\pm$  S.D. (4 replicates/condition). Transport in cells transfected with negative miRNA is set to 100%. Differences between the negative miRNA control and the miRNA mimics were analyzed by one-way ANOVA, followed by Bonferroni correction: \*  $P < 0.05$ .

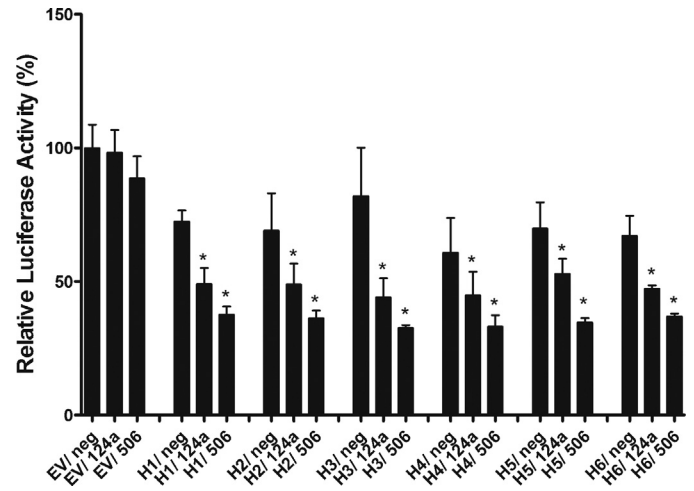
haplotypes. H3 was only present in Caucasians and African Americans, while H4, H5 and H6 were African American specific.

### 3.6. Effect of ABCC4 haplotypes on miRNA regulation

A luciferase reporter gene assay was used to measure the regulatory function of ABCC4 3'-UTR haplotypes. Six ABCC4 3'-UTR haplotypes were amplified or created by site directed mutagenesis and cloned into the pGL3-promoter vector downstream of luciferase. Empty vector or an ABCC4 3'-UTR haplotype luciferase vector and a *Renilla* transfection control vector were co-transfected with negative control miRNA, miR-124a or miR-506. Levels of luciferase activity relative to *Renilla* activity were measured 24 h post transfection. In the absence of miRNA mimics, ABCC4 3'-UTR haplotypes showed 0%–30% reduction in luciferase activity relative to empty vector (Figs. 6 and 7), suggesting a dynamic effect of endogenous miRNAs on the ABCC4 3'-UTR. Addition of miR-124a and miR-506 further reduced the luciferase activity of all six ABCC4 3'-UTR haplotypes, to ~35%–50% of empty vector (Fig. 6). None of the polymorphisms/haplotypes reversed the inhibitory effect of miR-124a and miR-506.

### 3.7. Specificity of ABCC4 regulation by miR-124a and miR-506

To further test the specificity of observed miRNA regulation of the ABCC4 3'-UTR putative binding site, sequences for miR-124a



**Fig. 6.** Effect of miR-124a and miR-506 on relative luciferase activity of ABCC4 3'-UTR haplotypes. HEK293T/17 cells were transfected with pGL3 promoter empty vector (EV) or plasmids containing ABCC4 3'-UTR haplotypes (H1–H6) and *Renilla* luciferase vector as a transfection control. Transfected cells were treated with 50 nM negative miRNA, miR-124a or miR-506 and results from one representative experiment ( $n = 3$  independent experiments) are presented as mean  $\pm$  S.D. (3 replicates/condition) relative to negative miRNA controls (set to 100%). Differences in luciferase activity with the different treatments were analyzed by one-way ANOVA, followed by Bonferroni correction: \*  $P < 0.05$ .

and miR-506 binding (located between base pairs 260–269 of ABCC4 3'-UTR) were mutated in H1 and H2 reporter plasmids (Fig. 1). Empty vector, H1, H2, mutated H1 and mutated H2 luciferase reporter plasmids and *Renilla* transfection control vector were co-transfected with negative control miRNA, miR-124a or miR-506. Levels of luciferase activity relative to *Renilla* activity were measured 24 h post transfection. miR-124a and miR-506 had no effect on mutated plasmids (Fig. 7) confirming specific binding of these miRNAs to the ABCC4 3'-UTR.

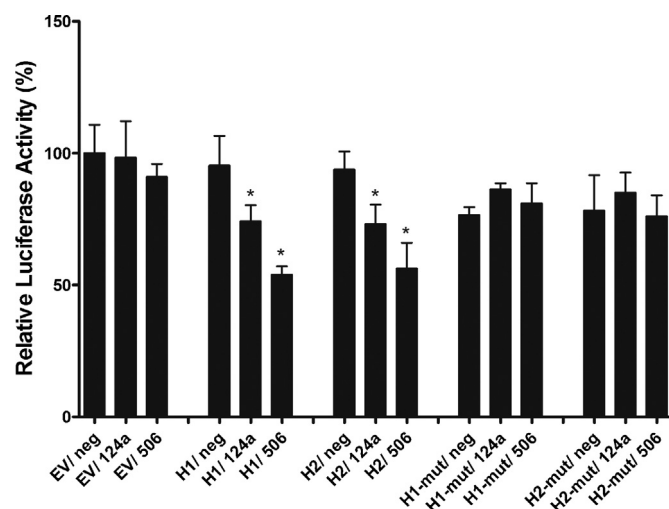
### 3.8. Correlation of MRP4 protein expression with miR-124a and miR-506 levels in human kidney samples

To test *in vivo* regulation of ABCC4 by microRNAs, protein and total RNA were extracted from 26 human kidney samples and MRP4 protein and miR-124a and miR-506 levels were measured. Expression of both miR-124a and miR-506 were negatively correlated with MRP4 protein expression (Fig. 8). This correlation was strongest for miR-124a expression and MRP4 protein (Pearson's correlation coefficient  $-0.62$ ,  $P = 0.007$ ). These results provide strong evidence for the regulation of MRP4 expression by miR-124a, and to a lesser extent miR-506, in human kidney.

**Table 1**

ABCC4 3'-UTR haplotypes and frequencies in three ethnic populations. ABCC4 3'-UTR haplotypes were inferred in Caucasians, African Americans and Asians using PHASE II software and HapMap and Pharmacogenomics of Membrane Transporters (PMT) genotyping data. Six haplotypes (H1–H6) were inferred with a frequency of more than 5% and nucleotide differences and population frequencies for Caucasians (CA), African Americans (AA) and Asians (AS) are shown.

SNP											Haplotype frequency		
rs3742106	rs4148551	rs4148553	rs9516521	rs1059751	PMT6316	rs9516520	rs9584273	rs16950472	rs9516519	rs34559063	CA	AA	AS
T > G	A > G	G > A	A > G	T > C	C > A	A > G	G > A	T > C	A > C	C > T			
H1		A		C							0.45	0.17	0.5
H2	G	G								T	0.36	0.37	0.46
H3			G			G			C		0.12	0.05	0
H4		G						C			0	0.11	0
H5		G			A					T	0	0.06	0
H6			A	C			A				0	0.05	0



**Fig. 7.** Effect of disruption of putative binding site for miR-124a and miR-506 on ABCC4 3'-UTR function. HEK293T/17 cells were transfected with pGL3 promoter empty vector (EV), plasmids containing ABCC4 3'-UTR haplotypes 1 or 2 (H1 or H2) or ABCC4 3'-UTR haplotypes 1 or 2 mutated at their putative miR-124a/506 binding site (H1-mut and H2-mut) and *Renilla* luciferase vector as transfection control. Transfected cells were treated with 50 nM negative miRNA, miR-124a or miR-506 and results from one representative experiment ( $n = 3$  independent experiments) are presented as mean  $\pm$  S.D. (3 replicates/condition) relative to negative miRNA controls (set to 100%). Differences in luciferase activity were analyzed by one-way ANOVA, followed by Bonferroni correction; \*  $P < 0.05$ .

#### 4. Discussion

The current study identified miR-124a and miR-506 as direct negative regulators of ABCC4. Using available *in silico* tools, eight miRNAs were predicted to bind ABCC4 3'-UTR, but only miR-124a and miR-506 were confirmed to downregulate endogenous expression and function of MRP4 protein *in vitro*. Reporter gene assays confirmed that miRNA targeting of ABCC4 3'-UTR was specific as disruption of their putative binding sites on ABCC4 resulted in rescue of the phenotype. Moreover, we observed a negative correlation between miR-124a and miR-506 with MRP4 protein expression in human kidney, suggesting *in vivo* significance of our *in vitro* findings.

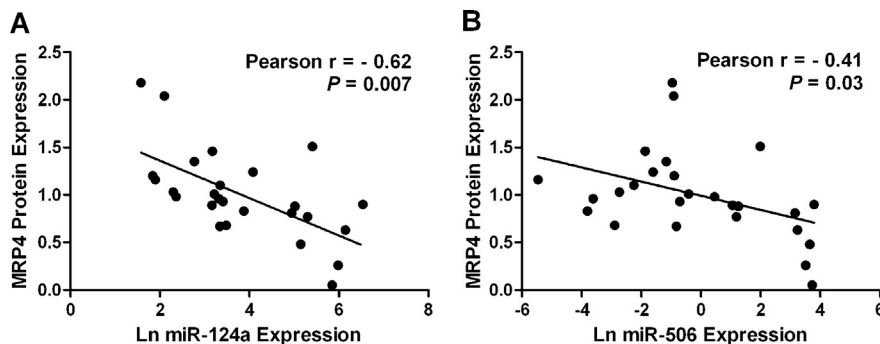
Over the past decade, knowledge of gene regulation by miRNAs has rapidly evolved but there is still a very limited understanding of miRNA regulation of genes involved in drug response [37]. Isolated reports on ABC transporters regulated by miRNAs indicate a critical need for research in this area. ABCB1 (MDR1) expression

is regulated by miR-27a and miR-451 [26] and by miR-200c [38]. ABCC1 (MRP1) expression is inversely correlated with miR-326 expression in a panel of advanced breast cancer tissues, suggesting a role for this miRNA in MRP1 regulation [28]. Also, ABCC2 (MRP2) expression is inversely correlated with miR-379 in cells treated with rifampicin. miR-379 was confirmed to bind the ABCC2 3'-UTR and reduce its expression [29]. ABCG2 (MXR) regulation by miRNAs has been better studied and miR-328, miR-519c and miR-520 h bind to ABCG2 3'-UTR and reduce its expression [30,32,39,40].

There is a recent report investigating regulation of a broad range of ABC transporters in cancer tissues by miRNAs [34]. ABCA1 was regulated by miR-101 and miR-135b, ABCC1 by miR-26a, miR-145, miR-199a, miR-199b and miR-296, ABCC5 by miR-101, miR-125a, let-7a and let-7b, ABCC10 by let-7a and let-7b, and ABCE1 by miR-26a, miR-135b and miR-145. ABCC4 regulation was investigated in the same study and three miRNAs, namely miR-125a, miR-125b and miR-143, were found to downregulate ABCC4. Interestingly, in the current study miR-143 was also predicted by *in silico* tools to bind ABCC4 mRNA 3'-UTR but failed to regulate endogenous MRP4 protein expression in HEK293T/17 cells and was not investigated further. This discrepancy could be explained by different experimental systems used to test regulatory miRNAs. On the other hand, the previous study did not identify miR-124a and miR-506 as potential ABCC4 regulators. Liver tissue was used to identify regulatory miRNAs, but miR-124a is predominantly expressed in peripheral blood mononuclear cells, brain and kidney and miR-506 is predominantly expressed in testicles, adrenal glands and at low levels in kidney [41,42], and therefore these miRNAs were not relevant in the previous study in liver.

Both miRNAs identified as ABCC4 regulatory elements in our study share an almost identical seed sequence at the 5' end but differ in their 3' ends (Fig. 1). miR-124a is repeatedly implicated in neurogenesis [43] and in glioblastoma and leukemia biology [44,45]. Interestingly, MRP4 was also implicated in leukemia biology and shown to influence leukemic cell maturation through regulation of intracellular cAMP levels [46]. There is also evidence for MRP4 being upregulated in human leukemic cells resistant to 6-mercaptopurine [47]. Previous data and our findings provide the basis for more extensive investigation of cross talk between ABCC4 and miR-124a in normal blood cells and leukemia.

miR-506 was cloned relatively recently and is implicated in cancer cell proliferation and growth [48,49]. There is also evidence for up regulation of miR-506 in kidney allografts with fibrosis [50]. Further studies are needed to elucidate potential cross talk between ABCC4 and miR-506 in testicles and kidneys, where both ABCC4 and miR-506 are expressed.



**Fig. 8.** Correlation of miR-124a and miR-506 levels with MRP4 protein expression in human kidney samples. Levels of (A) miR-124 and (B) miR-506 (relative to geometric mean of RNU6B and RNU48) and MRP4 protein (relative to GAPDH) were correlated in 26 human kidney samples. MRP4 data are presented relative to the mean of all samples and microRNA data are natural logarithm transformed to obtain a normal distribution. Spearman correlation coefficients were calculated for transformed data and correlations with corresponding  $P < 0.05$  were considered significant.

Recently, SNPs in the miRNA regulatory pathways (miRSNPs) were classified as a novel class of functional polymorphisms present in the genome [51]. miRSNPs include SNPs affecting miRNA biogenesis and SNPs in or near target miRNA binding sites. The *ABCC4* 3'-UTR is very polymorphic with several variants present at a minor allele frequency close to 50%. Six major 3'-UTR haplotypes at a frequency greater than 5% were inferred with at least two polymorphisms present in each haplotype. None of the polymorphisms were located within or in proximity to the predicted binding sites for miR-124a and miR-506 and had no effect on the regulation of *ABCC4* and *MRP4* expression by these miRNAs.

In conclusion, *ABCC4* is directly regulated by miR-124a and miR-506, polymorphisms in the *ABCC4* 3'-UTR have no significant effect on this miRNA regulation and the expression of these miRNAs is correlated with *MRP4* levels in human kidney. Further investigations are needed to elucidate the clinical significance of our findings and to characterize the regulation of *ABCC4*/*MRP4* expression by miR-124a and miR-506 in additional tissues.

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